A RATIONAL APPROACH TO THE PROBLEM OF CANCER CHEMOTHERAPY [1]

L. V. HEILBRUNN AND W. L. WILSON

Department of Zoology, University of Pennsylvania, Philadelphia, Pa.; Department of Physiology and Biophysics, University of Vermont, Burlington, Vt.; and the Marine Biological Laboratory, Woods Hole, Mass.

In spite of the fact that over a long period of time, many workers in various parts of the world have been studying the curative effect of various chemical agents on cancer, there has been no very great effort to determine why these agents act as they do, and there is at present but little pertinent information as to the type of action these agents have on the cancer cell.

The problem is complicated by the fact that many of the very agents that have a definite curative effect can also act as carcinogenic agents. This was first noted by Haddow in 1935, and he and his collaborators have written a number of papers on the subject. In his very useful book on the "Biochemistry of Cancer," Greenstein (1954) frequently refers to this phenomenon as the "Haddow paradox." But neither Haddow nor Greenstein has any interpretation of the paradox. In 1951, Haddow in writing about carcinogens and substances which have a therapeutic effect on cancer writes (p. 264): "But in no case—a striking fact—do we know the place in the cell at which they act—whether the cell surface, the cytoplasm, the nuclear membrane, the nucleus itself—or the nature of the receptors with which they combine." And all that Greenstein has to suggest when he considers the problem is that (p. 278) "The capacity for intellectual flexibility combined with scientific care is one of the demands in the field."

In the past, most of the work that has been done on the chemical treatment of cancer and the theory back of such treatment has been done by chemists. And whatever efforts they have made toward interpretation have for the most part been inspired by chemical concepts built around the idea of some disturbance of metabolism. But some of the most powerful chemotherapeutic agents do not affect the growth of the cell. Thus in the presence of nitrogen mustard or its oxygen derivative, Nitromin, cells increase in size but do not divide (Bodenstein, 1947; Friedenwald, Buschke and Scholz, 1948; Sato, Belkin and Essner, 1956).

When a tumor arises in an organ or tissue the appearance of the neoplasm is always accompanied by a great increase in the number of cells that are dividing. Thus for example in the brain, there are normally no mitoses, but in a brain tumor there are great numbers of dividing cells. It is of course possible to believe that some increase in metabolism, or some change in metabolism, is the primary cause that started the tumor to develop. But it is just as possible to assume that the primary factor is an initiation of mitosis and that metabolic changes are a result rather than a cause.

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At the present time, we have a considerable body of information concerning the initiation of cell division, and there is also a satisfactory theory as to why and how cells can be made to divide. Moreover we have information as to the suppression of cell division and the reasons for it. Both the initiation and the suppression of cell division can be understood in terms of the behavior of the protoplasmic colloid, and the changes that occur in it that lead to the formation of the mitotic spindle. For a survey of this knowledge, see Heilbrunn's "Dynamics of Living Protoplasm" (1956). One of the strange facts about protoplasm is that the same agents which suppress its activity can also under certain conditions arouse it to activity. We thus have a parallel to the Haddow paradox, and indeed the initiation of cell division can be regarded as one example of the response to stimulation.

Can we hope therefore to interpret the chemotherapy of cancer and the Haddow paradox on the basis of a colloidal theory? Surely such an approach is worth looking into, especially in view of the fact that other types of interpretation have not as yet been successful.

In a long series of papers, most of them referred to in the book already cited, Heilbrunn and his co-workers have investigated the problem of the initiation and suppression of cell division from the standpoint of the colloidal properties of protoplasm and the changes that the protoplasmic colloid must undergo in order to form a mitotic spindle. The basic colloidal reaction of protoplasm is a clotting reaction similar in many respects to the clotting of blood. The protoplasm is in a state of equilibrium between the various factors which favor or induce clotting and those which prevent it. Before a mitotic spindle can form, a gelation must occur in the protoplasm. This is the mitotic gelation, and it can be induced either by a release of calcium from a bound state in the outer cortex of the cell or by the entrance into the cell of thrombin-like substances. Many agents prevent the mitotic gelation—among them, substances of a heparin-like nature.

Starting out from this point of view, we thought to investigate the action of certain chemotherapeutic agents which have been used in the experimental study of cancer as well as in clinical practice. The first question to be answered is whether or not these agents prevent the mitotic gelation. Then later we will consider the question of the Haddow paradox.

Materials and Methods

The cell we used as a test object was the egg of the worm *Chaetopterus pergamentaceus*. The eggs of this worm can readily be obtained at Woods Hole during the summer months. When the eggs are shed into sea water they are in the germinal vesicle stage. In 7 minutes (at 21° C.) the germinal vesicle breaks down and the first maturation division proceeds as far as the metaphase. Then all mitotic activity ceases until the egg is fertilized. Following fertilization the first maturation division is completed and the second maturation division immediately follows the first. As a result two polar bodies are given off and the egg then prepares for its first cleavage division. The mitotic spindle for this division appears at forty minutes after insemination (at 21° C.) and 50% of the eggs cleave at 56 minutes after insemination. Before the appearance of the mitotic spindle, the viscosity of the protoplasm increases markedly; this constitutes the mitotic gelation. Simultaneously with the appearance of the spindle the protoplasm becomes more fluid again so that

at the metaphase the viscosity is again low. Details of these changes are given by Heilbrunn and Wilson (1948). One advantage in using the *Chaetopterus* egg is that in any given lot the course of events in different individual eggs varies but little, and there is almost perfect synchrony.

Viscosity measurements were made with a hand centrifuge. At the present time it is not possible to buy suitable hand centrifuges. The ones we use are made for us by Mr. J. A. Appenzeller, technician of the Zoology Department of the University of Pennsylvania. They are adapted from a hand centrifuge sold by Sears, Roebuck and Co. and intended for the separation of cream from milk. In order to use these cream separators for our purposes it is necessary to fit them with a head which will hold glass tubes. The tubes we use have an outside diameter of 4 or 5 millimeters. When our centrifuges are turned at the rate of one turn of the handle per second, they give a force approximately 9,000 times gravity. We prefer to turn the handle once every two seconds; this gives a force one-fourth as great, that is to say, 2,250 times gravity. With a force of this magnitude, for most of the time between insemination and cleavage, it requires 7 seconds to move the granules in the *Chaetopterus* egg sufficiently so as to give the impression of zones. (The heavier granules move centrifugally and the lighter granules centripetally.) The number of seconds required to produce zoning is taken as an arbitrary viscosity value. At about 27 minutes after insemination (at 21° C.) the viscosity of the protoplasm begins to increase, and by about 30 or 32 minutes the viscosity has increased until it is approximately twice what it was before this mitotic gelation began. The viscosity then stays high until the spindle appears, a matter of about 8 minutes. It is during this time that tests of viscosity must be made if we are to discover if a given substance keeps the protoplasm fluid and prevents the mitotic gelation. These tests must therefore be made rapidly so that it is not possible to obtain definite values. But we can be sure that in the controls mitotic gelation has occurred if the viscosity is high enough so that it requires more than 8 seconds of centrifugal turning to cause an appearance of zones. Actually the viscosity during normal mitotic gelation is 14 in our arbitrary units. In the tables we record the viscosity of the control eggs as more than 8; it almost certainly is 14.

The nitrogen mustard used in our experiments was obtained from Sharp and Dohme in the form of a commercial preparation called Mustargen. This preparation comes in separate sealed vials, each of which contains 10 mg. of nitrogen mustard, that is to say methyl-bis(beta-chloro-ethyl) amine hydrochloride plus 100 mg. of NaCl. The contents of each vial were hastily dissolved in sea water, but because of the presence of the NaCl it was not possible to prepare solutions of high concentration, for such solutions would have been hypertonic and might have masked the effect of the nitrogen mustard.

Through the kindness of Dr. Edward S. Essner we were able to obtain Nitromin, an oxide derivative of nitrogen mustard manufactured by the Yoshitomi Pharmaceutical Industries of Osaka in Japan and distributed by Takeda Pharmaceutical Industries, also of Osaka. Nitromin is methyl-bis(beta-chloroethyl)amine-N-oxide hydrochloride. This compound is less toxic than nitrogen mustard and has been claimed to have better therapeutic value.

In using 6-mercaptopurine, we had difficulty. This substance is scarcely soluble at all in sea water. In order to obtain a solution we dissolved it first in a small amount of normal NaOH. Then strongly acidified sea water was added until a pH

TABLE I

Effect of Mustargen on fertilized Chaetopterus eggs

%	pH	Viscosity at 32–38 min.	% Cleavage
0 (control)	7.5	>8	94
0.1		8 or less	18
0.05		8 or less	20
0.025		8 or less	16
0 (control)	7.5	>8	94
0.1		8 or less	27
0.05		8 or less	32
0.025		8 or less	19

a little higher than that of sea water was reached. At this pH, microscopic observations showed the solution to be full of suspended material, so that we could not be at all certain as to how much of the substance remained in solution. In all of our experiments we were dealing with a saturated solution of unknown concentration, and we have the impression that in sea water 6-mercaptopurine is barely soluble.

RESULTS

When nitrogen mustard is dissolved in sea water the resultant solution has a much lower pH than does sea water. In our first experiments we made no attempt to neutralize the acid in our solutions, and the results of these experiments were therefore discarded. Table I gives the results of two experiments in which the Mustargen solution was made more alkaline by the addition of NaOH solution. In both experiments, the solutions were brought to a pH of 7.5 and were thus still somewhat less alkaline than sea water. However, in preparing dilutions from the 0.1% solution of nitrogen mustard, the dilutions were of course made with sea water so that in the lower concentrations of the drug, the pH was not very different

TABLE II

Effect of Nitromin on fertilized Chaetopterus eggs

%	pH	Viscosity at 31–35 min.	% Cleavage
0 (control)	7.9	>8	100
0.4		8 or less	0.5
0.3		8 or less	4
0.2		8 or less	9
0 (control)	8.0	>8	97
0.5		8 or less	0
0 (control)	7.8	>8	97
0.4		8 or less	0
0.3		8 or less	0
0.2		8 or less	2

from that of sea water. Moreover, a pH of 7.5 has but little effect on the protoplasm of *Chaetopterus* eggs.

The experiments with nitrogen mustard indicate that this substance keeps protoplasm fluid and prevents the mitotic gelation. It thus acts in the same way as do various other antimitotic substances previously studied by us (Heilbrunn and Wilson, 1950a, 1950b, 1956; Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954). The mechanism of this action will be discussed later.

Our experiments with Nitromin gave more striking results than those with nitrogen mustard. Like Mustargen, Nitromin when dissolved in sea water causes a substantial reduction in the pH. As before, we added enough NaOH to bring back the solution to a pH like that of sea water. The results obtained with Nitromin are shown in Table II. They show conclusively that this derivative of nitrogen mustard keeps protoplasm fluid and completely prevents the mitotic gelation. Very few of the eggs exposed to rather dilute solutions of Nitromin ever cleave.

As pointed out in the section on Materials and Methods, 6-mercaptopurine is very sparingly soluble in sea water, and sometimes we wondered if any of it went

TABLE III

Effect of 6-mercaptopurine on Chaetopterus eggs

%	pH	Viscosity at 30–35 min.	% Cleavage
0 (control)		>8	97
0.1	8.25	8 or less	49
0 (control)		>8	98
0.3	8.1	8	99
0.3 (exposure 30 min. before fertilization)	8.1	8	70

into solution at all. Our results with this substances are not very impressive; they are shown in Table III. The results we did obtain indicate clearly enough that 6-mercaptopurine tends to keep the protoplasm of the *Chaetopterus* egg fluid. This effect is more pronounced when the solution is a little more alkaline, presumably because at the higher alkalinity more of the substance stays in solution. Also at the higher pH the inhibition of cleavage was greater.

Nitrogen mustard, Nitromin and 6-mercaptopurine thus all have the same sort of effect on the protoplasmic colloid. All of them tend to keep the protoplasm fluid and prevent the mitotic gelation. In the past it has been shown many times that the same agents which prevent gelation may, in other concentrations, have quite the opposite effect (for references and discussion, see Heilbrunn, 1956). Fat solvent anesthetics, which keep protoplasm fluid and thus prevent response to stimulation, may in certain concentrations act as stimulating agents and when they do they induce a clotting or gelation of the protoplasm. Now it is proper to consider the prevention of cell division by agents which do not kill the cell as a form of anesthesia or narcosis, and indeed various anesthetic agents do prevent cell division. And the initiation of cell division can be regarded as a response to stimulation. All this being true, might it not be possible to show that with other concentrations of nitrogen

TABLE IV

Effect of ethyl urethane on fertilized Chaetopterus eggs

% Urethane	Viscosity 30–33 min. after fertilization	% Cleavage
0 (control)	>8	100
2	8 or less	0
1.5	8 or less	0
1	8 or less	0

mustard or Nitromin a gelation of the protoplasm could be induced and perhaps also an initiation of cell division? If we could show this, we would have a way of interpreting the Haddow paradox.

We did not attempt to do this experiment with nitrogen mustard, for the preparation of this drug that was available to us—Mustargen—contains ten times as much NaCl as it does nitrogen mustard, and if we made relatively concentrated solutions we would arrive at concentrations of salt which would in themselves cause the initiation of cell division. However, with Nitromin this difficulty does not exist. Accordingly, we tried the effect of a 1% and a 0.5% solution of Nitromin on unfertilized *Chaetopterus* eggs. Both of these solutions caused a marked increase in the viscosity of the protoplasm. In the weaker solution this increase (after 80 minutes) was at least two-fold; in the stronger solution the viscosity increase was even greater and the protoplasm seemed quite solid. In both cases the drug caused a vacuolization of the protoplasm. This is the type of reaction which Loeb (1913) called cytolysis, and it is a reaction commonly produced by agents which initiate division in marine eggs when these agents are used in too strong a concentration or for too long an exposure. However, in the one experiment we tried, we were not able to obtain any initiation of cell division. In this experiment the eggs were exposed to 1% and to 0.5% Nitromin for periods of 1, 2, 5, 10, 20, 30 and 60 minutes. Our failure to obtain initiation of cell division with the Nitromin solutions was not surprising, for although in every case when egg cells are stimulated to divide, the viscosity of the protoplasm in the interior of the cell is markedly increased, the reverse is not true; for an agent which tends to gel or clot the protoplasm may be too toxic to permit cell division to proceed. Thus in the work on Nitromin, there is only a partial explanation of the Haddow paradox. For though it is true that Nitromin can produce opposite effects on the protoplasmic colloid, we know only that it can suppress cell division and not that it can initiate it.

TABLE V

Effect of ethyl urethane on unfertilized Chaetopterus eggs

Time of exposure to 3% urethane	Viscosity
20 min.	11
32	13
54	13
Control (untreated)	8

TABLE VI

Effect of 3% ethyl urethane in initiating cell division of unfertilized Chaetopterus eggs

Exposure time in minutes	Exp. A: % cleavage after 3½ hours	Exp. B: % cleavage after 6 hours	Exp. C: % cleavage after 8 hours
5	0	0	3
10	0	16	24
15	0	16	50
20	15	40	55
25	26	80	50
30	37	88	45
35	26		29
40	20		34
45	11		20
50	13		23
55	10		22
60	4		6
Control	0	0.05	9

We thought therefore to try the effect of urethane; for this substance, which is known to act both as a carcinogen and as a chemotherapeutic agent for tumors, is presumably less toxic than Nitromin. In relatively weak concentrations, ethyl urethane suppresses cell division in the *Chaetopterus* egg. This is shown in Table IV. This table also shows that in concentrations which suppress cell division, the urethane keeps the protoplasm fluid and prevents the mitotic gelation.

Higher concentrations of urethane have quite the opposite effect. Thus when unfertilized eggs are placed in a 3% solution of urethane, the protoplasmic viscosity increases sharply, as is shown in Table V.

Moreover exposure to 3% urethane can, in a high percentage of cases, cause the egg cells to divide. We have done some experiments of our own to show this, but experiments done by Mr. Herbert Schuel are more complete than ours and we prefer to present them. They are shown in Table VI.

DISCUSSION

Clearly, then, one and the same agent in different concentrations can cause either initiation of cell division or suppression of cell division and these opposite effects are readily correlated with the action the reagent has on the colloidal state of the protoplasm in the interior of the cell.

The facts as we have reported them are so clear cut that they scarcely require additional comment. Therapeutic agents commonly used in the treatment of cancer can prevent cell division by keeping the protoplasm fluid. Some of these agents, when used in different concentrations, can have opposite effects both on the physical state of the protoplasmic colloid and also, in the case of urethane, on the end result. Here, then, we have a way of interpreting the Haddow paradox, and we are able to supply the information Haddow was so concerned about, namely on which part of the cell these agents act and what they do.

But the question immediately arises as to the mechanism of the paradoxical ac-

tion. In the case of urethane, the answer is rather obvious in the light of what we know concerning the way various anesthetic or narcotic agents act on protoplasm. This subject is discussed at some length by Heilbrunn (1956). Suffice to say here that these agents liquefy the cortical protoplasm and release calcium from it; the calcium thus released enters the interior of the cell and there causes a clotting reaction, which may lead either to excitation or to a complete vacuolization of the protoplasm and death of the cell. But fat solvent anesthetics not only tend to free calcium from the cortex, they also tend to prevent calcium in the cell interior from causing a clotting reaction. There is a large and growing body of evidence in support of these statements (see Heilbrunn, 1956). In addition it should perhaps be noted that ether, which in low concentrations keeps the protoplasm of sea urchin eggs fluid (Heilbrunn, 1920, 1925), can in higher concentrations induce cell division (Mathews, 1900; McClendon, 1910); in these higher concentrations it causes a clotting reaction in the protoplasm.

It is to be hoped that other investigators will join with us in approaching the problem of cancer chemotherapy from the standpoint of the colloid chemistry of protoplasm. At the present time there are so many excellent and well trained workers interested in the metabolic approach, and scarcely anyone concerned with the reasons why the protoplasmic colloid changes in such a way as to form a mitotic spindle. And yet, without mitosis there can be no cancer, and if there are relatively non-toxic ways of preventing mitosis, certainly this is a field that should be investigated on a large scale. Insofar as we know at present, neither the initiation nor the suppression of mitosis depends on any particular metabolic pathway and it certainly does depend on colloidal changes in the protoplasm. Antimitotic substances such as can be extracted from ovaries can indeed be used to cure mice inoculated with a lethal ascites tumor (Heilbrunn, Wilson, Tosteson, Davidson and Rutman, 1957) and in their therapeutic action on this tumor they are at least as effective as nitrogen mustard or Nitromin. Indeed more recent experiments have shown them to be decidedly more effective.

Summary

1. Nitrogen mustard, Nitromin, 6-mercaptopurine, and urethane suppress cell division in *Chaetopterus* eggs.

2. This inhibition of mitosis is due to the fact that these agents keep the protoplasm fluid and prevent the mitotic gelation.

3. In relatively high concentration, both Nitromin and urethane cause a gelation of the protoplasm and in these concentrations, urethane can initiate cell division in a high percentage of the eggs.

4. An interpretation is given of this paradoxical action of reagents in causing either liquefaction or gelation, either suppression or initiation of cell division.

5. The results are believed to provide an explanation of the Haddow paradox.

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